Sperm storage and antioxidative enzyme expression in the honey bee, *Apis mellifera*

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Abstract

Honey bee (*Apis mellifera*) sperm remains viable in the spermatheca of mated female honey bees for several years. During this time, the sperm retains respiratory activity, placing it at risk of the damaging effects of reactive oxygen species common to many biological processes. Antioxidative enzymes might help reduce this damage. Here we use quantitative real-time RT-PCR to establish gene-expression profiles in male and female honey bee reproductive tissues for three antioxidative enzymes: catalase, glutathione-*S*-transferase (GST) and superoxide dismutase (SOD1, cytosolic). Catalase and GST showed ten- to twenty-fold transcript increases in the sperm storage organs of mated queens vs. unmated queens, whereas SOD1 levels are high in both mated and unmated queens. Male reproductive and somatic tissues showed relatively high levels of all three antioxidant-encoding transcripts. All three enzymes screened were higher in mature males vs. young males, although this effect did not appear to be confined to reproductive tissues and, hence, need not reflect a role in sperm longevity. Furthermore, antioxidative enzyme transcripts remained present, and apparently increased, in male tissues long after sperm had matured and seminal fluid was produced. We also found measurable levels of catalase transcripts in honey bee semen. The presence of catalase transcripts in both reproductive tissues and semen in bees suggests that this enzyme might play a key role in antioxidative protection.

Keywords: sperm longevity, reproduction, social insect, transcription.

Introduction

The process of sperm storage in insects can be viewed on two scales, the initial movement of sperm to the sperm storage organ (spermatheca) and the subsequent longevity of sperm once in the spermatheca. Substantial work in the fruit fly, *Drosophila melanogaster*, has focused on the former of these, and several individual proteins from the seminal fluid appear to be important in the safe transport and packaging of viable sperm (reviewed by Chapman, 2001; Wolfner, 2002). One protein, Acp36DE, is secreted in the accessory gland of male *Drosophila*, then is delivered to females at mating via the seminal fluid. Knockout experiments indicate that this protein plays a key role in the delivery of sperm to the spermatheca (Neubaum & Wolfner, 1999). A second seminal fluid protein, Acp62F, has been suggested as a sperm preservative owing to its role as a protease inhibitor, although this function has not been tested directly (Lung *et al.*, 2002). Additional male-derived *Drosophila* accessory proteins impact such diverse features as female behaviour, antibacterial defence, sperm competition and oogenesis (Wolfner, 2002), and the group as a whole shows other traits indicative of a role in mating system dynamics, such as positive sequence-level selection (Swanson *et al.*, 2001). Female-derived proteins probably also play a role in *Drosophila* sperm storage, although we know of no studies confirming such a function.

Drosophila is arguably a poor system in which to determine the effects of paternal and maternal proteins on long-term survival of sperm, because sperm storage generally is limited in these flies to a period of several weeks. By contrast, female social insects in the order Hymenoptera (ants, bees and wasps) store sperm for years, and even decades in the case of some ants (Holldobler & Wilson, 1990). Consequently, they face intense selection to store sperm efficiently and reliably from early mating events. In fact, queens of many social insect species produce no reproductive offspring whatsoever for several years after colony founding, meaning that they (and their mates) will succeed in a reproductive sense only if stored sperm remains viable for this entire period. Understanding the extreme measures needed to maintain viable sperm in social insects should

clarify both general mechanisms of sperm preservation in insects and mechanisms unique to the evolution of insect colonies. Natural sperm storage mechanisms in honey bees (*Apis mellifera*) have an additional applied importance, because the exploitation of these mechanisms could help breeding programmes and efforts to store and maintain genetically diverse lines (Collins, 2000). Despite these strong motivations, genetic mechanisms behind sperm storage in honey bees and other social insects remain unexplored.

Here we determine expression profiles in honey bee reproductive and somatic tissues for members of one likely class of sperm preservatives, the antioxidative enzymes. Antioxidative enzymes can increase sperm longevity by reducing levels of damaging reactive oxygen species (ROS). ROS, including hydroxyl and hydroperoxyl radicals, as well as hydrogen peroxide, are produced by enzymatic and non-enzymatic reactions during biological processes (Pardini, 1995), and are known to impact survivorship of sperm from diverse organisms (Ball *et al.*, 2000; de Lamirande & Gagnon, 1992). Because honey bee sperm are stored in an aerobic environment and retain some metabolic activity during storage (Blum & Taber, 1965; Koeniger, 1986), they most likely face some level of oxidative stress throughout storage. Antioxidative enzymes superoxide dismutase (SOD), catalase and glutathione-*S*-transferase (GST) have been proposed to reduce oxidative risk during sperm storage in bees (Weirich *et al.*, 2002). SOD converts superoxide anions to H_2O_2, which is removed in part by catalase activity. In many eukaryotes, GSTs act by catalysing the attachment of ROS and other toxic molecules to glutathione, a first step in their removal from the body. A role for antioxidants in sperm longevity has been validated for several vertebrates *in vitro* (Ball *et al.*, 2000; Bilodeau *et al.*, 2002). In honey bees, one attempt was made to determine the effects of catalase on honey bee sperm motility (Verma, 1978) and respiration *in vitro* (Verma, 1981), although impact on longevity was not directly tested. A role for antioxidative enzymes in aiding sperm survival *in vivo* is even less clear. In fact, we have found no studies that have tested the role of antioxidants on natural sperm survival in insects.

Recently, Weirich *et al.* (2002) documented variable levels of antioxidative enzyme activity in female reproductive and somatic tissues in honey bees, in a fashion that suggests an antioxidative role in sperm storage. They showed, for example, that mated honey bee queens exhibit higher activities of catalase and GST in the spermatheca than do unmated queens. They also documented catalase and SOD activity in honey bee sperm and seminal fluid, respectively. Several questions remain with respect to the generation of antioxidative enzymes in honey bee reproductive tissues. First, are antioxidative enzymes found in mated queens as a result of paternal (e.g. seminal fluid or sperm itself) contributions or maternal synthesis? Is the continued presence of spermathecal antioxidative enzymes over queen lifetimes a result of queen production and, if so, from which tissues? Finally, because the antioxidative mechanisms for these enzymes differ, is there evidence for differential expression depending on different oxidative risks during spermatogenesis vs. sperm storage? We measured transcript levels for catalase, GST and cytosolic SOD in somatic and reproductive tissues of immature and mature queens and males. We find evidence suggestive of roles for both female- and male-derived antioxidants in sperm storage and show that transcripts for these enzymes increase in activity as males and queens become reproductively mature.

Results

Expression in queen tissues

Catalase transcripts were found in each of the queen tissues screened. Transcript levels in the spermatheca differed significantly between unmated and mated queens of identical age (x, the transcript level relative to the riboprotein control, $= 0.019$, SE $= 0.002$ vs. $x = 0.15$, SE $= 0.057$) and between each of these and 1-year-old mated queens ($x = 0.681$, SE $= 0.19$). Catalase transcripts also were higher in the muscle tissue and ovaries of older queens than in these tissues for young, unmated queens (Fig. 1). GST transcripts in the spermatheca differed between young, unmated queens, and both of the mated queen groups (e.g. $x = 0.009$, SE $= 0.006$ in unmated vs. 0.139, SE $= 0.070$ in 21-day mated, and 0.059, SE $= 0.019$ in older mated queens), with no apparent effect of queen age. SOD1 did not change in expression with mating status, nor with the age of mated queens (Fig. 1). Nevertheless, SOD1 showed higher transcript amounts than the other two antioxidants across most tissues. In muscle, SOD1 levels had a mean x of 0.38 (SE $= 0.077$), 0.21 (SE $= 0.042$) and 0.69 (SE $= 0.16$) in unmated, young and older queens, respectively. Most strikingly, spermathecal levels of SOD1 transcripts in unmated queens were substantially higher than those of the other enzymes in this tissue ($x = 0.71$, SE $= 0.14$, *t*-test, $P < 0.0001$).

All three enzymes showed similar transcript levels between the spermatheca and the spermathecal gland (Fig. 2), although six of six comparisons showed a trend toward higher expression in the gland vs. spermatheca (sign test, $P < 0.05$). As with the combined extracts from these tissues, above (Fig. 1), there was an age-based increase in transcript level for catalase (Wilcoxon rank-sum test, $P < 0.05$). For spermathecal glands, catalase showed a ten-fold increase in transcript amount between 21-day-old and 1-year-old mated queens ($x = 0.19$, $n = 5$, and $x = 2.38$, $n = 6$, respectively, Wilcoxon rank-sum test, $P < 0.01$). GST and SOD1 transcript levels did not change significantly with age in these two tissues, although power to resolve
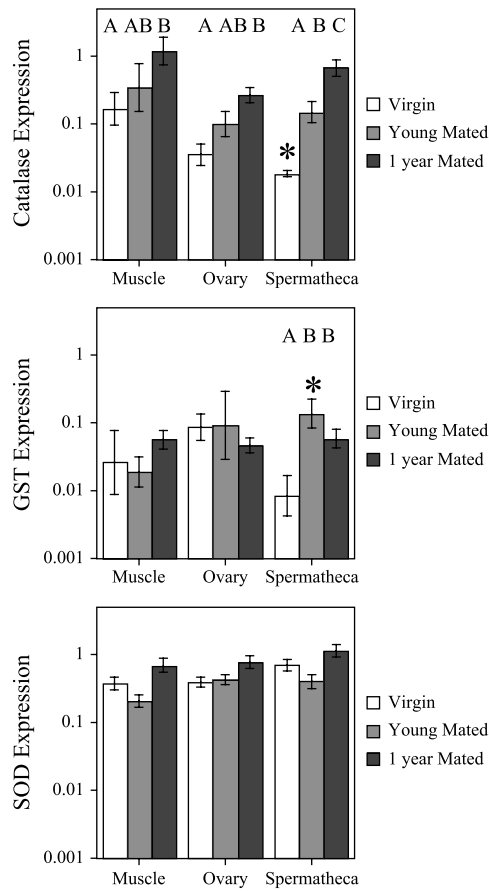
Figure 1. Relative transcript number for antioxidant-encoding genes in unmated queens and mated queens aged 21 days or 1 year; the *x*-axis is on $\log_{10}$ scale, $\pm$ SE. Letters A, B and C refer to age or mating-status differences within a tissue. *Significantly different from the expression level in muscle control. Sample size for each tissue in younger queens was 8, in older queens 16.

Figure 2. Relative transcript number for antioxidant-encoding genes in spermatheca and attached gland, for young (21-day-old) and old (1-year-old) queens. *Significant difference between ages by Wilcoxon rank sum test ($P < 0.05$). Six paired gland and spermatheca samples were analysed for each age.

differences was low because of high variation across the few samples scored (Fig. 2).

Expression in male tissues

Male bees showed significantly higher average transcript levels for all three enzymes, when compared with queens. For example, muscle transcripts of the gene encoding catalase were 12.2 times those of the control riboprotein in 21-day-old males (SE = 1.75), whereas they averaged only 0.16 (SE = 0.10), 0.35 (SE = 0.31) and 1.19 (SE = 0.56) for unmated queens and young and old mated queens, respectively. Transcripts were present in each of the reproductive tissues screened at levels similar to those found for muscle (Fig. 3). Mucous glands showed significantly lower transcript levels for catalase in 7-day-old males vs. testes in the same males (*t*-test, $P < 0.05$). All other tissue-specific comparisons were non-significant within age classes. Although not significant for individual tissues, transcripts increased over developmental age for the antioxidative
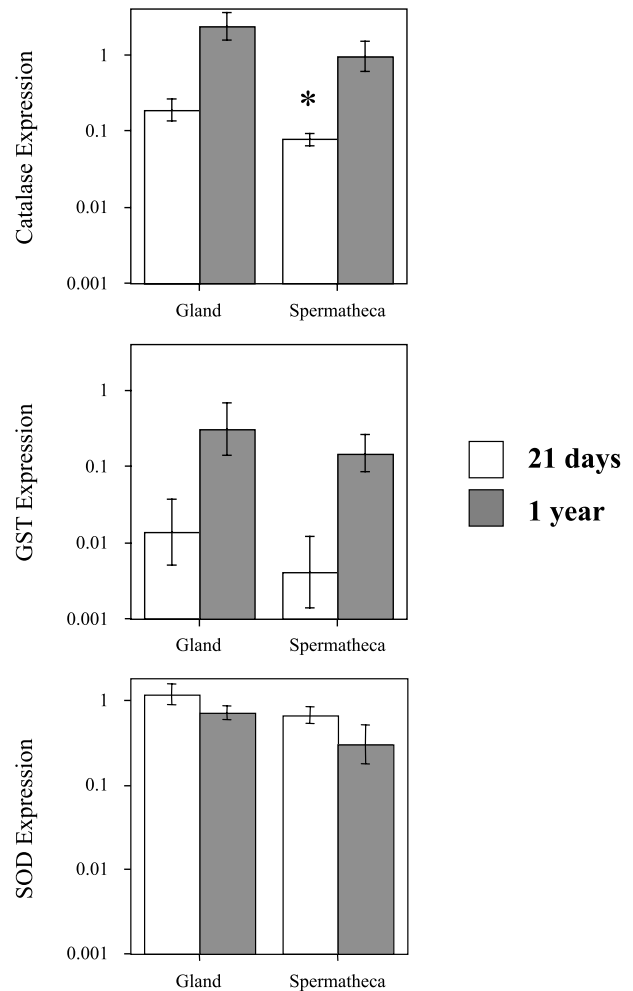
enzymes as a whole (e.g. sign test, 15/15 increased, $P < 0.001$). We found measurable transcripts of catalase and SOD1, and the RPL8 control in seminal fluids collected directly from males. In ten semen samples, catalase products tended to be higher than the control ($x = 1.6$, SE = 0.46) and SOD1 products were approximately 55% those of the control ($x = 0.55$, SE = 0.18).

Discussion

Reproductive tissues of honey bee queens differed substantially across queen types in transcript levels for catalase and GST, in a way that suggests a role for these enzymes in sperm storage. Within the sperm storage organ, both enzymes showed significantly higher levels in mated vs. unmated queens (Fig. 1). For catalase, transcript levels also increased with queen age. Older, mated queens
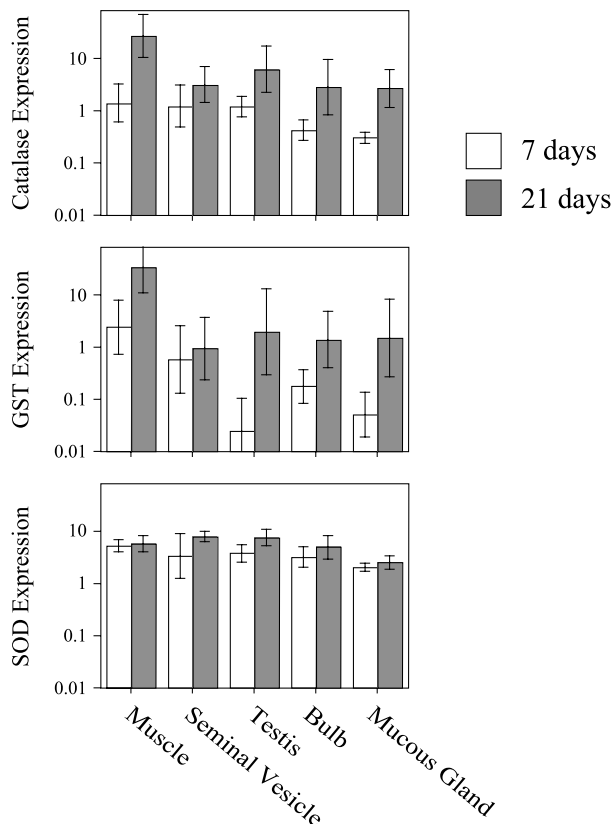
Figure 3. Relative transcript number for antioxidant-encoding genes in male muscle and reproductive tissues, for 7-day-old and 21-day-old bees. Nine samples were analysed for each tissue and age.

showed a ten-fold higher catalase transcript level than younger, mated queens. For GST, these differences were significant between mated queens and unmated queens, but not between the two age classes. Both the spermatheca and its attached gland emerged as potential sources of antioxidative enzymes involved with sperm storage. The ovary and muscle tissues also showed higher catalase transcript levels in older, mated queens vs. young, unmated queens. Although we did not directly quantify transcript levels, transcripts of catalase and SOD1 were approximately as abundant as a strongly expressed riboprotein control in tissues of mature queens.

Results from male tissues are equivocal with respect to a role for antioxidative enzymes in sperm longevity. Males showed generally higher transcript levels for the three antioxidants than did females (e.g. nearly ten-fold higher for catalase in the muscle tissue). These levels are consistent with higher protein activity levels (Weirich *et al.*, 2002). Interestingly, higher transcript levels occurred in both somatic (muscle) and reproductive tissues of males. It is also apparent that males maintain high expression of antioxidant-encoding genes even after the products of these genes seem likely to impact sperm or seminal fluids. Male

honey bees mature over a period of approximately 7 days after emergence (eclosion) as adults. Sperm is produced in the testes before eclosion, then migrates to the seminal vesicle from 3 to 8 days after eclosion, where it matures for several days (Koeniger, 1986). Mucous, which forms the bulk of the ejaculate in bees, is generally produced during the first 7 days after eclosion. Given this developmental order, it is somewhat surprising that transcripts in the testis, seminal vesicle and mucous gland are actually higher at 3 weeks of age than at 1 week. Because males invariably mate only once, if at all, and will commence mating flights within 2 weeks of eclosion, products required for successful mating are likely to be produced prior to 3 weeks of age. Consequently, for most males, it seems unlikely that the products of these late-expressed transcripts will be packaged with sperm or be incorporated into the seminal fluid. High antioxidative enzyme levels in males relative to queens might instead reflect a role in maintaining adult tissue and improving longevity. An especially high risk for oxidative damage in males seems unlikely, given their relatively short average lifespans (approximately forty-fold shorter than those of queens), and the fact that, with the exception of short-lived mating flights, males remain fairly quiescent in the colony throughout their lifespans. Queens, by contrast, maintain an extremely high reproductive output, laying eggs every few seconds during the reproductive season, for a net output of hundreds of thousand of eggs. Workers, whose average lifespan more closely approximates that of males, showed transcript levels similar to those of queens of the same age (J. D. Evans, unpublished data).

Our results were broadly consistent with measured enzymatic activity for these three antioxidants (Weirich *et al.*, 2002), with the exception of catalase muscle transcript levels in queens, which were substantial here despite minimal measured catalase activity in muscle tissue. It is conceivable that catalase transcripts or their protein products are not processed completely in the honey bee muscle, in which case they might be only poorly correlated with protein activity. Alternatively, these enzymes might be activated in response to behavioural or development cues not found for the bees in the previous study. Finally, the protein assays conducted by Weirich and colleagues measured only unbound catalase, resulting in a probable underestimate of catalase activity within cells.

These results provide the first molecular–genetic evidence that the environment of the spermatheca in a social insect changes upon mating in a way predicted to decrease oxidative stress on sperm. Our results, coupled with the results of Weirich *et al.* (2002), indicate that males can influence antioxidant levels as well, through catalase and SOD1 activity in the sperm and seminal fluids, respectively. It will be especially interesting to continue to explore the extent to which transcript levels of antioxidative enzymes themselves are packaged with honey bee sperm. In mammalian

systems, for example, mature spermatids retain gene transcripts and, presumably, are capable of translating these transcripts into active proteins (Trevino *et al.*, 2001). If this is possible in honey bee sperm, extended production of catalase and other antioxidants could help explain the great natural longevity of honey bee sperm in the face of oxidative risk (Collins, 2000).

Functional–genetic assays using RNA inactivation are now possible in honey bees (Beye *et al.*, 2002), allowing direct tests of the roles of antioxidative enzymes on sperm longevity. These assays could be applied to both queens and males to alter antioxidative enzyme levels in seminal fluids, sperm and the spermatheca. Sperm survivorship then could be assayed using secondary artificial insemination of sperm from queens with differing enzyme levels (e.g. Koeniger, 1986) or by direct screens of sperm viability (Collins & Donoghue, 1999; Collins, 2000). Given the unmatched longevity of sperm in the social insects, it will be of great interest to determine how their sperm-storage mechanisms differ from those found in the other eukaryotes.

Experimental procedures

Young Italian honey bee queens were obtained from a single commercial breeder (The Wilbanks Apiaries, Claxton, Georgia, USA), in April 2002. These queens, which were all approximately 21 days in age, fell into two groups. Half had been allowed to mate naturally and were ready to initiate egg laying, and a second group had been prevented from going on mating flights. A third group of mated honey bee queens (from Wilbanks Apiaries and a second apiary breeding the same strain in Georgia, USA) was collected after these queens had been laying eggs for one full year.

Male (drone) bees were reared in Beltsville (MD, USA) from the Wilbanks genetic stock. Frames with male pupae were taken from full-size colonies, and then were incubated at 34 °C for 6–12 h to provide emerging males of similar age. These males were marked, and then returned to their natal colony for either 7 days or 21 days prior to dissection.

Dissection and RNA isolation

Fresh queens were dissected directly in an RNA-stabilizing buffer solution (RNALater, Ambion, Austin, TX, USA) using a binocular microscope, forceps, insect pins and scissors. The exoskeleton was cut from thoraces and a section of flight muscle was removed and placed into 20 µl RNA lysis buffer (RNAqueous, Ambion). Abdomens were cut open and both tracheal and digestive materials were removed. Ovaries were cleared from other connective tissues and placed into 50 µl of the above lysis buffer. For eight unmated and twenty-eight mated queens, the spermatheca and associated gland were cut from the reproductive tract, removed as one and placed into 20 µl of lysis buffer. For an additional seventeen queens (eleven mated, six unmated), the spermatheca was separated from its attached gland and these tissues were placed separately into 20 µl of lysis buffer. After grinding the samples, volumes were brought to 250 µl lysis buffer and total RNA was extracted using the RNAqueous protocol and reagents, followed by elution in RNAse-inhibitory buffer.

Flight muscles were removed from male bees as in queens, above. The male reproductive tract was then dissected and separated into four components: the accessory (mucous) gland, the testis, the seminal vesicle and the ejaculatory bulb. Each tissue was placed separately into 100 µl of lysis buffer, after which total RNA was isolated as above, with final elution in 50 µl of Rnase-inhibitory buffer. Seminal fluid was collected from ten mature male bees through forced ejaculation. Approximately 1 µl of seminal fluid from each male (the average per-male contribution to mating) was placed into 200 µl of RNAqueous lysis buffer, after which RNA was extracted and eluted in 50 µl RNAse-free buffer.

RT-PCR

DNA contamination was removed from extracts using 10 U DNase I in a solution of 1 U RNAse inhibitor (Ambion) and incubation at 37 °C for 45 min. Complementary DNA was synthesized using 1 U Superscript II (Invitrogen, Carlsbad, CA, USA), 2 nmol DNTP mix, and a composite of 2 nmol poly dT-18 and 0.1 nmol poly dT(12–18). Synthesis was carried out at 42 °C for 1 h.

PCR primers for catalase and SOD1 (cytosolic, CuZn) were designed from sequenced clones present in a plasmid cDNA library derived from larval female honey bees (Evans & Wheeler, 2000; GenBank accession numbers AY462420, AY462419). These clones, 547 and 449 base-pairs in length, respectively, showed close matches with other eukaryotes for catalase and SOD1 (based on searches using BLAST-X in the NCBI nr database). They are 99% and 100% identical to complete catalase and SOD1 transcripts recently reported in GenBank (accession numbers AF436842 and AY329355, M. Corona and G. Robinson, University of Illinois). GST-2 was identified from an EST set derived from honey bee antennae (accession number BE844335, H. Robertson, University of Illinois). Ribosomal proteins RPL8 and RPS5, also isolated from a larval cDNA library (Evans & Wheeler, 2000; accession numbers BG101599 and BG101538), were used as controls for RNA concentration and amplification, because their expression is not expected to vary across tissues or life stages. PCR primer sequences used were: Amcat.F (5′–3′, = GGCGGCT-GAATTAAGTGCTA) Amcat.R (TTGCGTTGTGTTGGAGTCAT), AmSOD.F (AGCAGATGCAAGTGGTGTTG), AmSOD.R (GAGCACC-AGCATTTCCTGTAG), RobGST.F (CAATTTGATGAACGGGGAAC) and RobGST.R (GCCGTACCGATGTTTTTCGTA) for the three antioxidative enzymes. Ribosomal protein primers were AmRPL8.F (TGGATGTTCAACAGGGTTCATA), AmRPL8.R (CTGGTGGTGG-ACGTATTGATAA), AmRPS5.F (AATTATTTGGTCGCTGGAATTG) and AmRPS5.R (TAACGTCCAGCAGAATGTGGTA). PCR reactions consisted of forty cycles of 94 °C (1 min), 58 °C (1 min) and 72 °C (2 min), followed by a melt-curve analysis to confirm amplification of single-gene products.

Quantitative PCR on the cDNA products was carried out in 96-well plates using a real-time fluorescent reader (Icycler, Bio-Rad, Hercules, CA, USA), and the incorporation of the intercalating dye SYBRGreen I (Molecular Probes, Eugene, OR, USA). Reactions included 2 U *Taq* DNA polymerase with suggested buffer (Boehringer, Indianapolis, IN, USA), 0.2 µM fluorescein, 1 mM DNTP mix, 2 mM MgCl$_2$, 0.2 µM of each primer and a final concentration of 2.5 × SYBRGreen 1. Fluorescence was measured on multiple occasions for each sample during each 58 °C annealing stage. Values were normalized using the average per-sample fluorescence during cycles 2–10. From these normalized values, it was possible to determine the cycle at which each sample crossed a specific quantitative threshold (arbitrarily set at 10% of the

variance in expression across all samples for cycles 2–10). This threshold cycle was used for all comparisons within a single plate. PCR products also were visualized on agarose gels, to screen for possible DNA contamination. The genomic sequences for RobGST and RPL8 include introns of 520 bp and 100 bp, respectively, within the amplified regions, allowing size-based identification of any contaminated samples. Where identified, DNA-contaminated samples were excluded from all analyses. To avoid complications from interindividual (genetic or environmental) variation, individual bees for which one tissue sample showed DNA contamination were excluded altogether.

Data analysis

Because total RNA amounts varied significantly across tissues, expression levels were normalized by subtraction against the threshold cycle of ribosomal protein controls RPL8 and RPS5. cDNAs generated from fifty-two quantified RNA samples were used to define the relationship between threshold cycle number and cDNA amount in these controls (RPL8 threshold = 21.8 cycles − 1.48 * log(Total RNA); $r^2 = 0.74$, $P < 0.0001$). The exponential component of this relationship (e.g. 1.5) was then used to convert cycle number differences into relative amounts of cDNA for the antioxidant transcripts. RPL8 was the best correlate with RNA concentration across different samples, and so was used to normalize the samples for the described analyses. This gene is in the top 15% of genes expressed in honey bee larvae and adults, based on macro-array analyses (ranking nineteenth in a set of 179 genes; Evans & Wheeler, 2000).

For each tissue, differences between reproductive and age classes were compared by *t*-tests or non-parametric (Wilcoxon rank-sum) tests on the means. All comparisons were based on threshold cycle number itself, rather than transformed estimates of RNA quantity, because the former value best reflects experimental error. Accordingly, because the threshold cycle is an exponential function, negative standard errors are consistently smaller than positive standard errors.

Acknowledgements

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